**In vitro** biological activities of *Thunbergia laurifolia* stem and leaf with reference to rosmarinic acid

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In vitro biological activities of *Thunbergia laurifolia* stem and leaf with reference to rosmarinic acid

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Abstract

*Thunbergia laurifolia* Lindl. (Thunbergiaceae family) have been shown to be the natural antioxidant source to cope with oxidative stress. By DPPH and FRAP antioxidant assays, this study found that rosmarinic acid exhibited the highest antioxidant activity followed by leaf and stem ethanolic extracts of *T. laurifolia*. By intracellular antioxidation assay against H₂O₂-induced ROS in EA.hy926 endothelial cells using 2′,7′-dichlorodihydrofluorescein diacetate as a probe, all tested samples exhibited marginal protection i.e. *T. laurifolia* leaf extract (77.31 ± 0.66%), rosmarinic acid (88.10 ± 6.105%) and *T. laurifolia* stem extract (91.30 ± 0.86%) comparing to the control (100%). Cytotoxicity against 5 cancer and 2 normal cell lines were evaluated by MTT cell viability assay. Rosmarinic acid and the leaf extract inhibited cell viability selectively in each cell lines, whereas the stem extract revealed no cytotoxicity (IC₅₀ = >100 µg/ml) in all cell lines.

Keywords: *Thunbergia laurifolia*, rosmarinic acid, reactive oxygen species, antioxidant activity
1. Introduction

Reactive oxygen species (ROS) or simply called free radicals are the molecules containing reactive oxygen which can be found in all aerobic organisms. In normal physiological condition, these molecules are endogenously generated at low concentration to function in cellular signaling (Zhang et al., 2016). In contrast, high concentration of ROS as a result of the imbalance between ROS generation and degradation, is defined as oxidative stress. Oxidative stress is a serious pathological causative factor for numerous diseases due to its highly reactivity to protein, nucleic acids and lipid. Therefore, the more prolonged period of cells exposure to high concentration of ROS, the more severity of diseases and disorders are generated. Oxidative stress with high ROS level can be commonly found at the site of vascular inflammation which is one of the complications for vascular disorders and diseases such as atherosclerosis, ischemic heart disease, cerebrovascular disease and diabetes mellitus (Siti, Kamisah, & Kamsiah, 2015).

Plants containing polyphenols are considered as a natural source of antioxidants in prevention of oxidative stress (Ghasemzadeh & Ghasemzadeh, 2011). *Thunbergia laurifolia* Lindl. (Family Thunbergiaceae) is a woody climber plant which is commonly found in Asia. The plant is called “Rang Jued” in Thai and has been used in traditional Thai medicine for hundreds of years. Fresh or dried crude drug of leaf and bark can be individually prescribed to treat inflammation, fever and poisoning (Thongsaard & Marsden, 2002). There are numerous scientific reports on the leaf of *Thunbergia laurifolia* including antioxidation (Suwanchaikasem, Chaichantipyuth, & Sukrong, 2014), antiproliferation (Jetawattana, Boonsirichai, Charoen, & Martin, 2015), antidiabetes (Kosai, Jiraungkoorskul, & Jiraungkoorskul, 2015), detoxifying effect
(Chattaviriya, Morkmek, Lertprasertsuke, & Ruangyuttikarn, 2010), antinociceptive and anti-inflammatory effects (Boonyarikpunchai, Sukrong, & Towiwat, 2014).

Despite the medicinal properties of *Thunbergia laurifolia* stem, there is still lack of scientific reports on its biological activity. However, the phytochemical study of the aerial part of *Thunbergia laurifolia* indicated that some flavonoids and many antioxidant compounds were found as the constituents in the plant (Kanchanapoom, Kasai, & Yamasaki, 2002). Rosmarinic acid, a phenolic ester consisting of caffeic acid and 3,4-dihydroxyphenylacetic acid is one of the main phytochemical compound in *Thunbergia laurifolia* (Suwanchaikasem et al., 2014). This natural compound has been published for its remarkable biological and pharmacological properties in many scientific reports (Amoah, Sandjo, Kratz, & Biavatti, 2016).

In this work, the leaf and stem of *Thunbergia laurifolia* and its phytochemical constituent, rosmarinic acid were investigated for the free radical scavenging activity, reducing power and intracellular antioxidation. Additionally, cytotoxic effect against 5 cancer and 2 normal cell lines were also examined.

2. Materials and Methods

2.1 Chemicals and reagents

Rosmarinic acid, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), RpMI-1640 medium and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Missouri, USA). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from GIBCO (New Zealand). All chemicals and reagents used were of analytical grade.
2.2 Plant collection and preparation of extracts

*T. laurifolia* stems and leaves were collected in Thailand and authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. Plant voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Dried plant materials were pulverized and then extracted with ethanol in a Soxhlet apparatus to obtain exhausted ethanolic extracts. The obtained residues were evaporated *in vacuo* and stored at 4°C until further use.

2.3 DPPH radical scavenging assay

Fifty µl of different concentrations of extracts, rosmarinic acid and positive controls (ascorbic acid and BHT) in ethanol were added to 150 µl of 120 µM DPPH ethanolic solution. Then the 96-well plate containing the mixture was incubated in the dark at room temperature for 30 min and subjected to measure the absorbance at 517 nm. The test was done in triplicate. The inhibition percentage was evaluated following this formula:

\[
\% \text{ Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100
\]

2.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent solution was prepared in the ratio of 10:1:1 including 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-trlazine (TPTZ) solution and 20 mM ferric chloride respectively. Twenty-five microliters of the extracts, rosmarinic acid and positive controls (ascorbic and BHT) in ethanol were added to 175 microliters of FRAP reagent solution. Then the 96-well plate containing the mixture was incubated at room temperature for 30 minutes and subjected to measure the absorbance at 593 nm. The test was done in triplicate. Ferrous sulfate calibration curve was established to
evaluate the reducing antioxidant power expressing in mM of ferrous iron per milligram of the samples.

2.5 Intracellular antioxidative assay

EA.hy926 human umbilical vein endothelial cells from American Type Culture Collection (ATCC) were grown in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin and grown in the incubator with a humidified atmosphere containing 5% CO₂ at 37 °C. The subculture of confluent cells was done using 0.25% trypsin in sterile phosphate buffered saline (PBS). Firstly, cell viability under various concentrations of rosmarinic acid, hydrogen peroxide (H₂O₂) as well as T. laurifolia stem and leaf extracts were assayed.

MTT cell viability assay was slightly modified from previous study to evaluate cytotoxic effect according to the previous study with slightly modification (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987). Briefly, the cells were seeded into 96-well plate (1x10⁵ cells/ml) at 37 °C for 24 hr, thereupon, the cells were incubated with different concentrations of T. laurifolia (stem, leaf) extracts, rosmarinic acid and hydrogen peroxide (H₂O₂) for 24 hr. After removing the medium, MTT solution (0.4mg/ml) was added and incubated for 4 hr. Thereafter, MTT solution was removed and replaced by 100% DMSO. The resulting formazan was measured at 570 nm for IC₅₀. The further step was antioxidation assay against H₂O₂-induced intracellular ROS.

The assay was slightly modified from previous study to quantitate the amount of ROS using 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) which could diffuse into the cell and then enzymatically hydrolyzed by cytoplasmic esterase to produce dichlorodihydrofluorescein (DCFH₂) as a non-fluorescent and it could be rapidly oxidized to a fluorescent dichlorofluorescein (DCF) by intracellular ROS (Mutsuko,
Briefly, following the cell treatments, the cells were washed twice with cold-PBS and incubated with 5 µM of DCFH-DA for 30 min. The solution of DCFH-DA was removed and then the cells were washed twice with cold-PBS and incubated with 0.05 mg/ml of \( \text{H}_2\text{O}_2 \) for 30 min. The absorbance was measured with excitation at 485 nm and emission at 535 nm. The results were expressed as percentage of ROS which has direct proportion to DCF fluorescence.

2.6 Cytotoxic assay against cancer cell lines

WI-38 (human lung fibroblast), BT-474 (human breast ductal carcinoma), ChoGo-K-1 (human bronchogenic carcinoma), HepG2 (human hepatocellular carcinoma), KATO III (human gastric carcinoma) and SW620 (human colorectal adenocarcinoma) were purchased from American Type Culture Collection (ATCC). All cell lines were grown in RpMI-1640 media, supplemented with serum growth factor and antibiotic as aforementioned in the previous assay. All cell lines were grown to obtain the density of 1x10^5 cells/ml in 5% CO\(_2\) incubator at 37°C. The ethanolic extracts of *T. laurifolia* (leaf and stem), rosmarinic acid and doxorubicin (positive control) was subjected for cytotoxic determination using MTT cell viability assay as described in the previous part.

3. Results and Discussion

Oxidative stress is a deleterious key to contribute vascular dysfunction, which has been found to be the causative factor for several fatal diseases (Gracia, Llanas-Cornejo, & Husi, 2017). Presently, it was found that antioxidant obtained from natural sources is the potential compound to cope with oxidative stress (Lobo, Patil, Phatak, & Chandra, 2010). *T. laurifolia* has been used as the herb to treat inflammation, fever and poisoning. In previous study, rosmarinic acid, a polyphenolic phytoconstituent in *T.*
laurifolia has been proved to be the potential natural antioxidant (Suwanchaikasem et al., 2014). Furthermore, rosmarinic acid isolated from the ethanolic extract of T. laurifolia leaf showed the anti-inflammatory and anti-nociceptive properties in mice (Boonyarikpunchai, Sukrong, & Towiwat, 2014). The findings of this study demonstrated the bioactive potentials of the ethanolic extracts of T. laurifolia leaf and stem in regard to rosmarinic acid.

3.1 Effects of T. laurifolia extracts and rosmarinic acid on the in vitro antioxidant activities

The yields of ethanolic extracts of T. laurifolia leaf and stem were found to be 15.2 and 9.0 g/100 g, respectively. The results of all test samples for in vitro antioxidant activities were shown in Table 1. DPPH scavenging activity of all samples exhibited a dose-respond relationship (Figure 1). T. laurifolia leaf and stem extracts showed IC$_{50}$ of 151 and 195 µg/ml. However, rosmarinic acid showed better activity than ascorbic acid, having the IC$_{50}$ of 52 and 118.29 µg/ml, respectively. Reducing power of the antioxidant was expressed as the amount of ferrous sulfate ion obtained by FRAP assay. T. laurifolia leaf and stem extracts showed reducing power ability with FRAP values of 0.26 and 0.18 mM Fe(II)/mg extract, respectively. Rosmarinic acid exhibited the similar value as the positive controls, ascorbic acid and BHT with FRAP values of 0.46, 0.42 and 0.55 mM Fe(II)/mg extract, respectively. Previous report indicated that the aqueous extract of microwave-dried leaves of T. laurifolia exhibited the highest scavenging property comparing to fresh leaves and commercial T. laurifolia leaves tea with the IC$_{50}$ of 0.50, 0.99 and 0.64 mg/ml, respectively (Chan & Lim, 2006). The use of different solvents (water, ethanol and acetone) to extract T. laurifolia leaves also indicated that all of the leaf extracts possessed antioxidant activity. (Ratchadaporn
Oonsivilai, M. G. Ferruzzi, & S. Ningsanond, 2008). These studies concluded that the plant had antioxidant potency.

3.2 Effects of *T. laurifolia* extracts and rosmarinic acid on intracellular ROS antioxidation

EA.hy926 cells were subjected to be used as the study model to investigate intracellular ROS inhibition. This immortalized cell line is one of the most used among endothelial cell lines due to the better characterized permanent human vascular, especially the large vessel endothelium (Bouis, Hospers, Meijer, Molema, & Mulder, 2001). Cell viability was tested prior to investigate ROS inhibition and it was shown that *T. laurifolia* leaf and stem extracts, rosmarinic acid and H$_2$O$_2$ inhibited cell viability with IC$_{50}$ of 0.48, >1, 0.23, 0.05 mg/ml for EA.hy926 cells after 24 hr treatment, respectively (Figure 2). Then the IC$_{50}$ of H$_2$O$_2$ (0.05 mg/ml) was used to induce ROS production in EA.hy926 cells. The results were expressed in the percentage of ROS. At 1 mg/ml, all test samples exhibited the marginal intracellular ROS antioxidation. *T. laurifolia* leaf exhibited 77.31 ± 0.66% of intracellular ROS production in H$_2$O$_2$-induced oxidative stress in the cells, followed by rosmarinic acid (88.10 ± 6.10%) and the stem extract (91.30 ± 0.86%) (Figure 3). Another study on intracellular ROS inhibition in HepG2 cells using DCFH-DA assay indicated that the cells preincubated with 80% ethanol extract of *T. laurifolia* leaf at 800 µg/ml for 29 hr significantly decreased oxidative stress approximately 60% of cellular oxidative stress comparing to the control (Rocejanasaroj, Tencomnao, & Sangkitikomol, 2014). Similar work in different cell lines using human umbilical vein endothelial cells (HUVEC) pretreated with rosmarinic acid for 12 hr at 25-200 µM reduced intracellular ROS in a dose-dependent manner by 31-59%, respectively (Huang & Zheng, 2006). EA.hy926 cells
might not be suitable cell lines for testing rosmarinic acid and the extracts containing rosmarinic acid according to its cytotoxicity on this cell line.

3.3 Cytotoxic effects of *T. laurifolia* extracts and rosmarinic acid on cancer cell lines

Cytotoxicity was determined using MTT cell viability assay. The results were expressed as the half maximal inhibition concentration (IC\textsubscript{50}) on cell viability after the cells were exposed to the test samples. Cytotoxic potential against all 5 cancer cell lines as well as 1 normal cell line were shown mostly in *T. laurifolia* leaf extract. According to the National Cancer Institute (NCI) has set the criteria for cytotoxicity determination in plant extract and pure compound that the plant extract must have IC\textsubscript{50} lower than 20 \(\mu\text{g/ml}\), while the pure compound must have IC\textsubscript{50} lower than 4 \(\mu\text{g/ml}\) (Geran, Greenberg, MacDonald, Schumacher, & Abbott, 1972). Therefore, all test samples in this study were not effective against cancer cell lines (Table 2). Another report of four cancer cell lines tested with water, ethanolic and petroleum ether extracts of *T. laurifolia* leaf exhibited IC\textsubscript{50}>100 \(\mu\text{g/ml}\) against all the test cell lines indicating low cytotoxicity (Ratchadaporn Oonsivilai, Mario G. Ferruzzi, & Suwayd Ningsanond, 2008). Similar study revealed that rosmarinic acid decreased cell viability of HepG2 cell lines as its concentration increased in time-dependent manner with IC\textsubscript{50} of 33 ± 0.74 \(\mu\text{g/ml}\) after 48 hours of incubation (Ma et al., 2018). In this study, it was found that *T. laurifolia* ethanolic leaf extract was more toxic against ChoGo-K-1 than other cell lines (IC\textsubscript{50} 29.92 \(\mu\text{g/ml}\)). From this study, cytotoxic potential of *T. laurifolia* leaf against cancer cell lines as well as normal cell line was exhibited. The toxicity of *T. laurifolia* leaf should be further investigated.

4. Conclusions
T. laurifolia leaf and stem extracts as well as its phytoconstituent, rosmarinic acid were revealed their marginal potentials on in vitro cytotoxicity against cancer cell lines. Rosmarinic acid exhibited strong antioxidant activity in vitro via the DPPH and FRAP assays comparing to the positive control in each test.

Acknowledgments

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References


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Figure 1 DPPH scavenging activity of test compounds

Figure 2 Effects of T. laurifolia extracts, rosmarinic acid and H$_2$O$_2$ on cell viability of EA.hy926 cells determined by the MTT assay
Figure 3 Effects of *T. laurifolia* extracts and rosmarinic acid on the level of intracellular ROS in EA.hy926 cells using H$_2$O$_2$ as the ROS inducer
### Table 1 In vitro antioxidant activities of test samples

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<td>WI-38</td>
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<tr>
<td><em>T. laurifolia</em> leaf extract</td>
<td>45.30</td>
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<tr>
<td><em>T. laurifolia</em> stem extract</td>
<td>&gt;100</td>
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<td>Rosmarinic acid</td>
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<td>Doxorubicin</td>
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### Table 2 Cytotoxic activity (IC$_{50}$) against various cell lines

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<tr>
<td><em>T. laurifolia</em> stem extract</td>
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<td>Rosmarinic acid</td>
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